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The basic premise of this research is that the turnover of dopamine (DA) or other monoamines by monoamine oxidase (MAO) can place on oxidative stress on electron transport and respiration of mitochondria. In turn, damage to mitochondria can contribute to the progression of Parkinson's disease and to the damaging effects of environmental neurotoxins. Work conducted this past year supports the basic premise. We have shown that: (1) MAO activity suppresses both respiration and electron transport. (2) Formation of protein mixed disulfides (PrSSG) accompanies damage to mitochondria. (3) MAO inhibitors block both formation of PrSSG and mitochondrial damage. (4) MAO-A, which is the isoform of the enzyme in DA neurons, appears more active in these regards than MAO-B (glial enzyme). (5) In addition, we have developed a new and improved assay for measuring PrSSG. The new laboratory findings support the working hypothesis and help to clarify the pathophysiology of neurodegenerative mechanisms affecting DA neurons. Over the longer range, the new leads concerning thiol redox status (viz., oxidation of GSH, formation of PrSSG) can lead to improved methods to protect DA neurons from damage by environmental neurotoxins or from the ravages of Parkinson's disease.

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INTRODUCTION

This proposal addresses the general goals of providing improved understanding of the pathophysiology of neurodegenerative processes affecting dopamine-secreting neurons. The research focuses on oxidative stress and damage to mitochondria evoked by the action of monoamine oxidase, and encompasses developing information that could lead to new treatment strategies for delaying or preventing the progression of Parkinson's disease. The proposal deals reversible damage to mitochondrial electron flow based on the formation of protein-disulfide linkages with oxidized glutathione (viz., protein mixed-disulfides). As described in the original Statement of Work, the proposal, in general, deals with: (1) Monoamine oxidase in the outer membrane of mitochondria within dopamine neurons; (2) Mechanisms of damage by oxidative stress, specifically that associated with role of glutathione and protein thiols in modulating the basic respiratory function of neural mitochondria; (3) Mechanisms for reversal of mitochondrial damage, and; (4) Laying the basic groundwork for new therapeutic strategies to protect against environmental neurotoxins and to prevent further dopaminergic damage in Parkinson's disease. Significant progress was made for goals (1) and (2) during the first year.

BODY OF REPORT

ABBREVIATIONS USED:

dopamine (DA),
glutathione (GSH),
glutathione disulfide (GSSG)
monoamine oxidase (MAO),
Parkinson's Disease (PD),
protein thiol (PrSH)
protein mixed-disulfide (PrSSG)

OVERVIEW

This project deals with a new finding that the activity of monoamine oxidase (MAO), an outer mitochondrial membrane enzyme, alters electron flow at the inner mitochondrial membrane (Cohen, Farooqui, & Kesler, 1997). This observation was made with both rat brain and liver mitochondria.

Electron flow, coupled to consumption of oxygen (respiration), is necessary for the production of ATP. Therefore, an implication is that the natural turnover of the neurotransmitter dopamine (DA) by MAO can affect energy production (ATP formation) and cellular viability. Parkinson's disease (PD) is characterized by an unexplained loss of DA neurons that originate in the substantia nigra, a melanized region of midbrain. It is well established that PD is itself characterized by a defect in mitochondrial respiration that targets Complex 1 activity of mitochondrial electron chain. The defect in Complex 1 is evident from autopsy studies (e.g., Schapira et al., 1990; Schapira, 1999). The same defect characterizes PD evoked by exposure to the environmental toxin MPTP (1-methyl-4-phenyl-2,3,5,6-tetrahydropyridine). The basis for the defect in idiopathic PD has not yet been established. However, it is believed that both genetic factors (Polymeropoulos et al., 1997; Kitada et al., 1998) and environmental toxins (Gorrell et al., 1996) may be involved.

In our working hypothesis, DA represents an "endogenous" neurotoxin. Its enhanced turnover in PD (Hornykiewicz and Kish, 1986) represents an endogenous oxidative stress evoked by MAO. The same oxidative stress can be caused by exposure to environmental neurotoxins, such as MPTP. The basic neurochemistry that underlies susceptibility of mitochondria to oxidative stress is given by the following equations:

$$DA + O_2 \longrightarrow H_2O_2 + NH_3 + \text{3,4-diOH-phenylacetaldehyde} \quad \text{(Eqn. 1)}$$

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O \quad \text{(Eqn. 2)}$$

$$GSSG + PrSH \longrightarrow PrSSG + GSH \quad \text{(Eqn. 3)}$$

In this sequence, MAO produces hydrogen peroxide (H_2O_2, eqn. 1), an oxidative stressor. The peroxide is removed by the enzyme glutathione (GSH) peroxidase (eqn. 2); glutathione disulfide (GSSG) is formed in the process. The GSSG then reacts with thiol groups of proteins (PrSH, eqn. 3), forming protein mixed-disulfides (PrSSG). Many proteins, including the components of Complex 1, require thiol groups for their enzymatic function. Therefore, loss of essential thiols (PrSH) via formation of PrSSG can lead to loss in enzymatic function. However, the loss is reversible; both the formation and the reversal reactions are enzymatically catalyzed.

The main research targets for the 1st year of this grant were: (1) To determine if MAO activity suppresses mitochondrial respiration in concordance with the suppression of electron flow, (2) to determine if PrSSG levels rose simultaneously, and (3) to evaluate the actions of selective MAO-A and MAO-B substrates and inhibitors. . Progress was made in each of these critical areas. In addition, (4) we developed a new and rapid analysis for PrSSG, which will speed the work in this area. A manuscript concerning targets (1) and (2) has been accepted for publication (Cohen & Kesler, in press), while a second manuscript concerning the new method for measuring tissue PrSSG is currently in review for publication.

EXPERIMENTAL STUDIES: (Part I. Mitochondrial Studies):

** Note: Figures & figure legends can be found in the rear

1. Effect of MAO activity on mitochondrial respiration:

Mitochondrial respiration is normally divided into stages or states: State 4 is achieved by the addition of substrate alone, State 3 by substrate + ADP, and State 5 (uncoupled or maximally stimulated respiration) by the addition of FCCP (carbonylcyanide p-trifluoromethoxyphenyl-hydrazone). The respiratory control ratios (State 3/State 4) of freshly isolated mitochondria were in the range 5.5-7.0 with 5 mM pyruvate plus 5 mM malate as substrate. State 3 respiration was in the range 72-103 ng-atoms oxygen/min/mg protein.

Figure 1 shows results from experiments in which mitochondria were incubated with 500 μM tyramine, with and without MAO inhibitors, at 27°C for 15 min; 500 μM was chosen because this is the estimated level of monoamine neurotransmitter in the cytosol of catecholamine neurons (Cohen et al., 1997). In these particular experiments, tyramine was used in place of DA in order to avoid formation of quinoidal oxidation products; tyramine cannot form quinones. However, in separate experiments. DA produced similar effects as tyramine.

State 3 and State 5 respiration were measured with pyruvate/malate (5 mM each) as substrate. Tyramine is a mixed MAO-A/MAO-B substrate (Tipton et al., 1976); therefore, a mixture of clorgyline (MAO-A inhibitor) and pargyline (MAO-B inhibitor) was used to inhibit MAO.

State 3 respiration was suppressed by 32.8 ± 1.7% (mean ± SEM, n=6) and State 5 respiration by 40.1% ± 1.9% (n=11) ($p<0.001$) compared to the corresponding control samples without tyramine (Figure 1). Inclusion of the MAO inhibitors (clorgyline and pargyline, 2 μM each) fully protected the mitochondria ($p<0.001$). MAO inhibitors by themselves did not affect mitochondrial respiration (not shown).

Representative oxygen electrode tracings are presented in Figure 2. Normally, mitochondrial respiration and synthesis of ATP are coupled on demand to the presence of ADP. Figure 2 illustrates State 3 respiration upon the addition of ADP, and State 5 respiration after uncoupling with FCCP. Figure 2 also demonstrates the diminution in both State 3 and State 5 respiration after exposure to tyramine.

Isolated mitochondria are delicate and susceptible to loss in respiratory function during incubation procedures. Therefore, samples were evaluated for the change in respiration due to the incubation conditions alone (comparison of incubated vs. non-incubated controls). The experiments with tyramine were limited to 15 min in order to limit this form of mitochondrial damage. Incubation for 15 min at 27°C decreased respiration by 27.3% ± 0.7% for State 3 and 21.7% ± 1.5% for State 5 ($p<0.01$, n=5/group, 2 experiments). However, the data in Figure 1 are expressed as the effects of tyramine relative to incubated controls; therefore changes due to experimental conditions, unrelated to tyramine, cancel out. Nonetheless, the loss of a highly vulnerable fraction of respiratory activity may cause the effect of tyramine to be underestimated in Figure 1. The stock, concentrated suspension of mitochondria (15-20 mg protein/ml) in Mops buffer, held on ice, was stable and did not lose respiratory activity over the course of the experiments (3-4 hrs).

Conclusion: These studies illustrate the direct effect of MAO activity on mitochondrial respiration and support a major premise of our research. Clearly, diminished electron flow evoked by MAO (Cohen, Farooqui, & Kesler, 1997) is translated into diminished respiration. In turn, this mechanism would be expected to diminish energy (ATP) production, and could form a major component of the neuronal damage evoked by environmental neurotoxins and in idiopathic PD.

2. Comparison of mitochondrial electron flow and respiration

We previously described the effect of incubation with tyramine, dopamine, or benzylamine, on the ability of mitochondria to reduce MTT, a formazan dye, during electron flow (Cohen et al., 1997). Inhibition was observed when electron flow was initiated either at Complex I (pyruvate) or at Complex II (succinate); however, Complex I showed a greater susceptibility to damage. Figure 3 presents results of experiments in which inhibition of respiration and electron flow were directly compared. Tyramine had an identical effect on respiration and electron flow: Inhibition of respiration by 500 μM tyramine after 15 min was 24.6% ± 1.2%, while inhibition of electron flow was 24.7% ± 2.4%.

The respiratory data in Figure 3 were obtained in the presence of FCCP as an uncoupling agent. FCCP was also present in samples analyzed with MTT in order to make direct comparison between the two assays under comparable conditions. It is known that FCCP partially suppresses reduction of MTT by rat brain mitochondria (Liu et al., 1997). In our experiments, FCCP diminished MTT reduction by 39.2 ± 3.4% ($p<0.01$, $n=6$). However, direct comparison of experimental results with and without FCCP added after a 15 min exposure to tyramine indicated that inhibition of electron transport was 24.7% ± 3.0% in the presence of FCCP and 29.4% ± 1.6% in its absence (n=6/group). Therefore, the basic phenomenon was essentially the same whether or not FCCP was present. In additional experiments, results were similar when FCCP was added either prior to or after incubation with tyramine. Therefore, FCCP does not interfere with the assessment of damage to electron transport.

Conclusion: These studies confirm that similar effects are observed when either electron flow (MTT reduction) or respiration (oxygen consumption) are evaluated.

3. Formation of PrSSG:

Glutathione peroxidase functions in intact mitochondria to remove H_2O_2 (Cf., eqn 2). GSSG, formed as the result of detoxification of MAO-generated H_2O_2, can react with protein thiols (Eqn. 3) to form PrSSG (Reed, 1990). Disulfide linkages with protein would be expected to suppress thiol-dependent enzymatic activity, such as that exhibited by Complex I or pyruvate dehydrogenase. We studied the formation of PrSSG in mitochondria. The results of Figure 4 show that PrSSG levels increased rapidly and progressively during incubation of mitochondria with either tyramine or dopamine. The increase over corresponding controls after 15 min (the time at which respiration was measured) was greater than 10-fold. This observation is commensurate with secondary oxidative damage based on a change in the thiol status of proteins. MAO inhibitors completely suppressed PrSSG formation (not shown).

Conclusion: These studies confirm a major rise in PrSSG in association with disruption of mitochondrial function.

4. Evaluation of selective MAO-A and MAO-B substrates and inhibitors:

DA and tyramine are mixed substrates for MAO-A and MAO-B. Studies with these substrates have generally utilized a combination of an MAO-A inhibitor (clorgyline) and an MAO-B inhibitor (deprenyl, pargyline) to suppress damage by MAO. For example, Figure 1 showed that inhibition of respiration by tyramine was completely suppressed completely by a combination of pargyline and clorgyline.

In order to better evaluate MAO-A and MAO-B, selective substrates were chosen. Serotonin was used as a selective MAO-A substrate and benzylamine as a selective MAO-B substrate. The MAO inhibitors were used at concentrations that gave selective inhibition of the appropriate form of MAO (0.2 μM). Figure 5 shows that at a concentration of 500 μM, serotonin was more powerful that benzylamine as an inhibitor of electron flow. Damage to mitochondrial electron flow by benzylamine was blocked by deprenyl (selective MAO-B

inhibitor), but not by clorgyline (selective MAO-A inhibitor). In contrast, damage by serotonin was blocked by clorgyline, but not by deprenyl.

Conclusion: These results verify that mitochondrial damage caused by addition of amines is due to the action of MAO. Both MAO-A and MAO-B are active in this regard, but MAO-A has the more pronounced effect. It is noteworthy that MAO-A is the form of the enzyme within DA neurons.

5. Summary of Mechanism of Mitochondrial Damage and Protection:

Figure 6 is a schematic representation of the proposed mechanism for formation of PrSSG and inhibition of mitochondrial respiration. DA, tyramine, or other amines serve as substrate for MAO, which generates H_2O_2, inducing oxidation of GSH near the outer mitochondrial membrane. GSSG, formed by GSH peroxidase, diffuses to the inner membrane and forms PrSSG. The latter reaction is catalyzed by thioredoxins and protein disulfide isomerases. Formation of PrSSG interferes with essential enzymatic function of thiol-dependent enzymes, such as pyruvate dehydrogenase and NADH-cytochrome c reductase (Complex 1).

EXPERIMENTAL STUDIES: (Part II. Methodology for PrSSG):

6. Background to PrSSG assays:

As described above, this project is based, in part, on the deleterious effect of formation of PrSSG from PrSH (Cf., eqns. 1-3 & Figure 6) at the inner membrane of mitochondria. Measurement of PrSSG is difficult and labor-intensive. During the course of our studies, we accidentally discovered that splitting of GSH from PrSSG can be rapidly accomplished simply by treating a protein pellet with alkali at room temperature. This observation eliminates tedious processing with sodium borohydride and will speed aspects of this project.

A standard method for measuring PrSSG is that described by Akerboom and Sies (1981) and by Modig (1968). Tissue proteins are isolated by precipitation with perchloric acid (PCA) and rinsed to remove endogenous GSH and GSSG. Then, the isolated protein is subjected to reduction with sodium borohydride ($NaBH_4$) at neutral pH to reduce disulfide bonds and the liberated GSH is measured. We have used this method, coupled to detection of GSH with the enzymatic recycling method described by Tietze (1969). In the course of our studies, we discovered that GSH is quantitatively liberated from PrSSG by solubilization of isolated protein pellets in alkali (0.2 M NaOH) without a need for addition of $NaBH_4$. Alkali was used to fully solubilize the protein in order to ensure that reduction of disulfide linkages would be complete. With this new finding, assays for PrSSG can proceed without special precautions (related to the generation of hydrogen gas) and extra handling steps associated with the use of $NaBH_4$. In essence, simply dissolving the protein pellet in alkali can be followed by immediate assay of liberated glutathione.

The validity of the new procedure for measuring PrSSG was verified as follows:

7. Comparison of the standard and new procedures:

For convenience, we used samples of stored tissues in which PrSSG levels had become elevated during storage. In fresh tissues, PrSSG levels are low, which makes measurement difficult. The elevated levels in stored tissue facilitated assays and the comparison of methodology.

Figure 7 shows results of assays on rat brain. Results obtained by reducing PrSSG with $NaBH_4$ near neutral pH (procedure 1) (Akerboom & Sies, 1981) are compared to similar reduction carried out in 0.2M NaOH (procedure 2); in addition, the spontaneous release of GSH by 0.2 M NaOH alone (without borohydride; procedure 3) is also shown. The protein pellet was fully solubilized by alkaline solution (procedures 2 and 3), but remained as a suspension at neutral pH (procedure 1). A striking observation is that values observed after alkalinization in the absence of borohydride are not different from those with borohydride present: that is, liberation of GSH in alkaline solution took place spontaneously without a need to add $NaBH_4$ as an external reducing agent. Values with NaOH alone were actually slightly higher (106.2% ± 1.0%) than those obtained by reduction with $NaBH_4$ at neutral pH ($p<0.05$).

PrSSG assays were conducted with samples of stored rat liver and brain in order to compare values obtained with NaOH alone to NaOH plus $NaBH_4$ (Table 1). Results were similar whether or not $NaBH_4$ was added. These data show that $NaBH_4$ is not needed to obtain full release of GSH, and that the phenomenon is not restricted to brain tissue.

Table 1. Liberation of GSH from PrSSG by $NaBH_4$ in 0.2 M NaOH compared to 0.2 M NaOH alone ($NaBH_4$ omitted) for samples of stored rat brain and liver.

		μmoles GSH liberated/g tissue	
Tissue		$NaBH_4$/NaOH	NaOH alone
Brain	Expt. 1	0.870 ± 0.004	0.928 ± 0.019[a]
	Expt. 2	0.748 ± 0.013	0.730 ± 0.008
Liver	Expt. 1	2.588 ± 0.051	2.843 ± 0.024[a]
	Expt. 2	1.983 ± 0.053	2.040 ± 0.022

Values are the mean ± SEM for 4 individual samples per group.
[a] $p<0.05$ compared to $NaBH_4$/NaOH, 2-tailed Student t-test.

Conclusion: Processing with sodium borohydride is not required. GSH is quantitatively liberated from PrSSG in alkaline solution.

8. Kinetic parameters:

The speed of the reaction that liberates GSH from PrSSG in NaOH was assessed (Figure 8). Release at room temperature was remarkably rapid, yielding 50.3 ± 1.3% of maximum after only 10 seconds (the first data point).

Although the basis for PrSSG assays is that precipitation of proteins, followed by repeated sonication and recentrifugation in acidic solution, virtually eliminates free GSH from the protein pellet, the very rapid release of GSH on solubilization of the protein in alkali raised the possibility that considerable GSH was entrapped within the precipitated protein, rather than bound in a disulfide linkage. To address this issue, SDS (sodium dodecylsulfate, 1% w/v) was added to solubilize the protein at neutral pH and, then, the protein was reprecipitated with PCA and washed twice by sonication and recentrifugation. Any possible effects of residual SDS were excluded by measurements performed with internal standards of GSH (95% recovery), and by experiments in which diluted SDS (10,000-fold dilution) was added to standards (97% recovery). Comparison of GSH released from washed protein pellets directly solubilized in 0.2 M NaOH (10.4 ± 0.3 nmoles/mg protein, N=8) vs. samples previously solubilized with SDS (10.0 ± 0.5 nmoles/mg protein, N=7) yielded identical values (not significantly different, 2-tailed t-test). Therefore, the GSH released in alkaline solution represents bound glutathione and not free GSH entrapped within the protein pellet.

9. Comparison of PrSSG levels in fresh tissue to literature values.

Assays of fresh rat liver carried out by liberating GSH from PrSSG with 0.2 M NaOH (without $NaBH_4$) showed normal levels of 16.5 ± 2.1 nmoles/g tissue (mean ± SEM, n=4). These values compare favorably to published values of 24 ± 8 nmoles/g (mean ± SEM, n=4) reported by Higashi et al. (1985) and values in the range 15-30 nmoles/g observed by Sies et al. (1987) and Brigelius et al. (1983).

Conclusion: Results with the new procedure reproduce values for normal levels of PrSSG taken from the literature. This observation fully validates the new procedure.

10. Lack of effect of 0.2M NaOH on the stability of GSH.

GSH can undergo autoxidation to form GSSG. Because the enzymatic recycling procedure detects both GSH and GSSG, it would <u>not</u> be affected by conversion of GSH to GSSG. However, other methods, such as assays based on GSH-S-transferase or high performance liquid chromatography (Brigelius et al., 1983), would be negatively affected by loss of GSH through autoxidation. Therefore, we evaluated the autoxidation of GSH in 0.2 M NaOH. To measure GSH selectively, the direct reaction with DTNB at neutral pH was used. This required higher concentrations of GSH than those used in the enzymatic-recycling assay. Incubation of 200 μM GSH in 0.2 M NaOH for 1 h, followed by dilution to 15 μg/ml in neutral buffer and subsequent assay with DTNB showed no detectable loss in GSH (98.9% ± 2.6% of control, n=5). Similar results were obtained in an experiment conducted with 1 mM GSH (98.7% ± 2.6% of control, n=6). Therefore, the material liberated in alkaline solution is amenable to assays based on the reduced form of glutathione.

11. Mechanism for the spontaneous liberation of GSH from PrSSG in alkaline solution.

A likely mechanism for release of GSH from PrSSG in alkaline solution is via displacement by protein thiols (PrSH) in a thiol/disulfide interchange. In alkaline solution, thiol groups are ionized and the reaction is represented by the following equation:

$$PrSSG + PrS^- \rightarrow PrSSPr + GS^- \qquad \text{(eqn. 4)}$$

The presence of a relatively high content of protein thiol groups in crude pellets isolated by acid precipitation of tissues provides the necessary reducing equivalents to liberate GSH. In liver, for example, protein thiols are present at a concentration of 94 nmoles/mg protein (Bellomo et al., 1990), equivalent to about 9 μmoles/g tissue, which is more than sufficient to react with even the high levels of 2.0-2.8 μmoles PrSSG/g tissue observed in stored rat liver (Table 1).

Conclusion: The current study shows that the chemical reduction of disulfide bonds by externally added reducing agents, such as $NaBH_4$ or dithiothreitol, is not necessary for the liberation of GSH from PrSSG. Liberation of GSH can be achieved simply by dissolving the protein pellet in 0.2 M NaOH, followed by an assay method of choice. In our studies, we use a variant of the enzymatic recycling method based on GSSG reductase (Tietze, 1969), which offers high specificity and sensitivity.

METHODS: The described studies were based on the following methods:

Rat brain mitochondria were isolated from groups of 3 Sprague-Dawley rats (250-275g) by a minor modification of the method of Clark and Nicklas (1970). The isolation medium consisted of 5 mM Mops (3-(N-morpholino)propanesulfonic acid), containing 0.225 M mannitol, 0.075 M sucrose, and 1.0 mM EGTA, adjusted to pH 7.4 with KOH. Isolation was carried out in the cold at 15,800 x g for 10 min, followed by 15,000 x g for 30 min in the Ficoll gradient, and a final rinse at 15,800 g for 10 min. The isolated mitochondria were suspended in cold Mops buffer at a concentration of 15-20 mg mitochondria protein/ml and maintained in an ice bath until used. The yield was 3-4 mg mitochondrial protein per rat brain.

Incubations were conducted by dilution of an aliquot of the mitochondrial preparation to 0.5 mg or 1.0 mg mitochondrial protein/ml in the respiration buffer (pH 7.2), which consisted of 5 mM Hepes, 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, and 1 mM $MgCl_2$ with 0.5 mg bovine serum albumin/ml at 27°C. Incubations were carried out at 27°C in a volume of 1 ml in plastic tubes (12-ml) on a water bath with gentle shaking (48 oscillations/min) for 15 min. Samples were processed individually with immediate assessment of respiration and rotation among the experimental groups. Samples not incubated with MAO inhibitors received additions of the inhibitors after the incubation was complete, just prior to the measurement of respiration or electron flow. Each experiment consisted of 10-12 samples (3-4 samples per group).

Respiration was measured in a miniature 0.6 ml chamber system equipped with a magnetic stirrer and maintained at 27°C. Oxygen consumption was assessed with a Biological Oxygen Monitor (Yellow Springs Instrument Co.). Measurements were made sequentially after the addition of pyruvate/malate (5 mM each) (State 4 respiration), followed by 0.4 mM ADP (State 3) and, lastly, 10 μM FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone; State

5). Pyruvate initiates electron flow and respiration at Complex 1. In some experiments, ADP was omitted and State 5 respiration was measured directly. Respiratory activity of the stock mitochondrial preparation, which was held on ice, was well maintained and did not change over the course of 3-4 h.

Electron flow was measured by reduction of MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide). Assays were carried out with a modification (Cohen et al., 1997) of the method described by Berridge and Tan (1993), except that the respiration buffer was used as the medium. Samples were incubated in buffer containing pyruvate/malate (5 mM each) and MTT (0.42 mg/ml) for 5 min at 27°C and, then, the reaction was quenched by the addition of a lysing buffer consisting of 10% (w/v) sodium dodecylsulfate and 45% (v/v) dimethylformamide, adjusted to pH 4.7 with glacial acetic acid. Absorbance readings were taken in duplicate on a plate reader and reported as the difference in absorbance between 550 nm and 620 nm. Spectrophotometry was preceded by a shaking period of 99 s on the plate reader. Individual samples were expressed as a percent of the mean control value in the experiment.

PrSSG was measured with a modification of the method of Akerboom and Sies (1981). The liberated GSH was measured on the plate reader with a modification of the enzymatic recycling method of Tietze (1969). Details of a modified procedure are provided in the text of this report (Experimental Studies, Part II). Protein was measured by the method of Lowry et al. (1951) and was used to normalize data for both PrSSG and respiration, which were expressed per mg protein.

Data are expressed as the mean ± SEM. Statistical assessment was conducted by the Tukey-Kramer multiple comparison test or, where appropriate, by the 2-tailed Student t-test.

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KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that MAO activity suppresses mitochondrial electron flow.
- Demonstration that MAO activity suppresses mitochondrial respiration.
- Demonstration that both MAO-A and MAO-B contribute to mitochondrial damage, but that MAO-A has a more powerful effect.
- Demonstration that PrSSG formation underlies damage to mitochondrial function.
- Discovery that GSH is spontaneously and quantitatively liberated from PrSSG in alkaline solution.
- Development of an improved and simplified assay for PrSSG.

REPORTABLE OUTCOMES:

1. **Manuscript:** Cohen G & Kesler N (1999) Monoamine oxidase and mitochondrial respiration. J. Neurochem., in press.
2. **Manuscript:** Cohen G & Yakushin S, Reactivity and Assay of Protein-Glutathione Mixed Disulfides, in review for Analyt. Biochem
3. **Manuscript:** Cohen G, Oxidative Stress, Mitochondrial Respiration, and Parkinson's Disease, Ann. N.Y. Acad. Sci., in press.
4. **Manuscript & Poster:** Cohen G & Kesler N, Monoamine Oxidase (MAO) Inhibits Mitochondrial Respiration, Ann. N.Y. Acad. Sci., in press.
5. **Book Chapter:** Cohen, G (1999) Oxidative stress and Parkinson's disease, in: Reactive Oxygen Species in Biological Systems (D. Gilbert & C. Colton, eds.), Plenum Press, NY, pp. 593-608.
6. **(Abstract & Oral presentation)** Cohen G, Cell signaling and the sulfhydryl redox status, Symposium on Oxidative Stress & Cell Signaling Systems, 30th Ann. Mtg. Amer. Soc. Neurochem., New Orleans, LA, March 13-17, 1999.
7. **(Abstract & Poster presentation)** Cohen G & Kesler N, The link between monoamine oxidase and mitochondrial respiration, 5th Intern. Conf. Parkinson's Disease & Movement Disorders, New York, NY, October 10-14, 1998.
8. **(Abstract & Poster presentation)** Cohen G & Kesler N., MAO inhibits mitochondrial respiration, 5th Ann. Mtg. Oxygen Society, Washington, DC, Nov. 19-23, 1998.
9. **(Abstract & Poster presentation)** Cohen G & Yakushin S, Assay of protein-glutathione mixed disulfides, 5th Ann. Mtg. Oxygen Society, Washington, DC, Nov. 19-23, 1998.
10. **(Abstract & Poster presentation)** Cohen G & Kesler N., Monoamine oxidase and mitochondrial respiration, N.Y. Acad. Sci. Conference on Oxidative/Energy Metabolism in Neurodegenerative Disorders, New York, NY, March 19-22, 1999.

CONCLUSIONS:

The basic premise of this research is that the turnover of dopamine (DA) or other monoamines by monoamine oxidase (MAO) can place on oxidative stress on electron transport and respiration of mitochondria. In turn, damage to mitochondria can contribute to the progression of Parkinson's disease and to the damaging effects of environmental neurotoxins. Work conducted this past year supports the basic premise: (1) MAO activity suppresses both respiration and electron transport. (2) Formation of protein mixed disulfides (PrSSG) accompanies damage to mitochondria. (3) MAO inhibitors block both formation of PrSSG and mitochondrial damage. (4) MAO-A, the isoform of the enzyme in DA neurons, appears more active in these regards than MAO-B (glial enzyme). (5) In addition, we have developed a new and improved assay for measuring PrSSG. The new laboratory findings support the working hypothesis and help to clarify the pathophysiology of neurodegenerative mechanisms affecting DA neurons. Over the longer range, the new leads concerning thiol redox status (viz., oxidation of GSH, formation of PrSSG) can lead to improved methods to protect DA neurons from damage by environmental neurotoxins or from the ravages of Parkinson's disease.

FIGURE LEGENDS

Fig. 1. Mitochondrial respiration with pyruvate/malate (5 mM each) as substrate after incubation of samples either without (control) or with 500 μM tyramine for 15 min at 27°C. Where indicated monoamine oxidase inhibitors (MAOI) were present (2 μM clorgyline plus 2 μM pargyline) during the incubation procedure. MAO inhibitors were added to all other samples just prior to the measurement of mitochondrial respiration. For State 3 respiration, 0.4 mM ADP was added (n=6; 2 independent experiments); for State 5, 10 μM FCCP was added (n=11, 4 independent experiments). Statistical assessment was by ANOVA followed by the Tukey-Kramer multiple comparison test.
*$p<0.001$ vs. untreated control;
**$p<0.001$ vs. corresponding tyramine-treated samples without MAO inhibitors

Fig. 2. Representative oxygen electrode tracings in the presence of pyruvate plus malate after incubation at 27 °C for 15 min, both without (panel A, control) and with 500 μM tyramine (panel B). Where indicated, ADP (0.4 mM) and FCCP (10 μM) were added.

Fig. 3. Direct comparison of mitochondrial State 5 respiration and electron transport (MTT assay) after incubation of samples with and without tyramine (500 μM) for 15 min at 27°C (n=6, 2 experiments). The mean difference (550 nm minus 620 nm) for control samples in the MTT assay was 0.083 absorbance units. *$p<0.001$, 2-tailed Student t-test.

Fig. 4. Formation of protein mixed-disulfides (PrSSG) in mitochondria during incubation at 27°C with 500 μM tyramine (■) or 500 μM dopamine (▲), compared to control samples (●). Results are pooled from two experiments (n=4 per group). Basal levels of PrSSG at zero time were 29.8 ± 17.0 pmoles/mg protein. The elevation in PrSSG in samples incubated with tyramine or DA, compared to corresponding controls, was significant at all time points ($p<0.001$).

Fig. 5. Effect of selective MAO substrates and inhibitors on mitochondrial electron flow. Substrates were either benzylamine (MAO-B substrate) or serotonin (MAO-A substrate). Selective inhibitors were deprenyl (MAO-B) and clorgyline (MAO-A). Substrate concentrations were 500 μM. Selectivity for MAO-A or MAO-B was obtained with 0.2 μM concentrations of the inhibitors.

Fig. 6. Proposed scheme for the inhibition of mitochondrial respiration by MAO and tyramine. Abbreviations: OMM, outer mitochondrial membrane and IMM, inner mitochondrial membrane.

Fig. 7. Release of GSH from protein mixed disulfides by $NaBH_4$ at neutral pH (first bar) or in 0.2 M NaOH (second bar), compared to release in 0.2 M NaOH alone (no $NaBH_4$, third bar). A protein pellet was isolated from stored rat brain tissue by precipitation with PCA. The pellet was washed twice in PCA by sonication followed by centrifugation, and then it was treated as stated above. The protein pellet was subsequently reprecipitated and discarded, and the supernatant was taken for assay of GSH. Data are expressed as percent of the mean value for $NaBH_4$ at pH 7.3. Mean values are from 9-11 samples in 3 independent experiments. The mean levels of PrSSG in 3 experiments were in the range 8.5-11 nmoles GSH equivalents/mg protein.

Fig. 8. Time course for the release of GSH from PrSSG in protein pellets isolated from stored rat brain. Protein pellets were prepared as in Fig. 7. Following the addition of 0.2 M NaOH, samples were sonicated for 10 seconds to solubilize the protein pellet and, then, samples were acidified with 0.4 M perchloric acid (final concentration) either immediately (viz., at 10 sec) or after standing for 5-30 min. Following centrifugation, the protein pellet was discarded and the supernatant was taken for assay. Data are expressed as a percent of the mean value at 30 min. Results are the mean ± SEM of 12 samples from 3 independent experiments, except for 8 samples (2 experiments) for data at 20 min.

Fig. 1

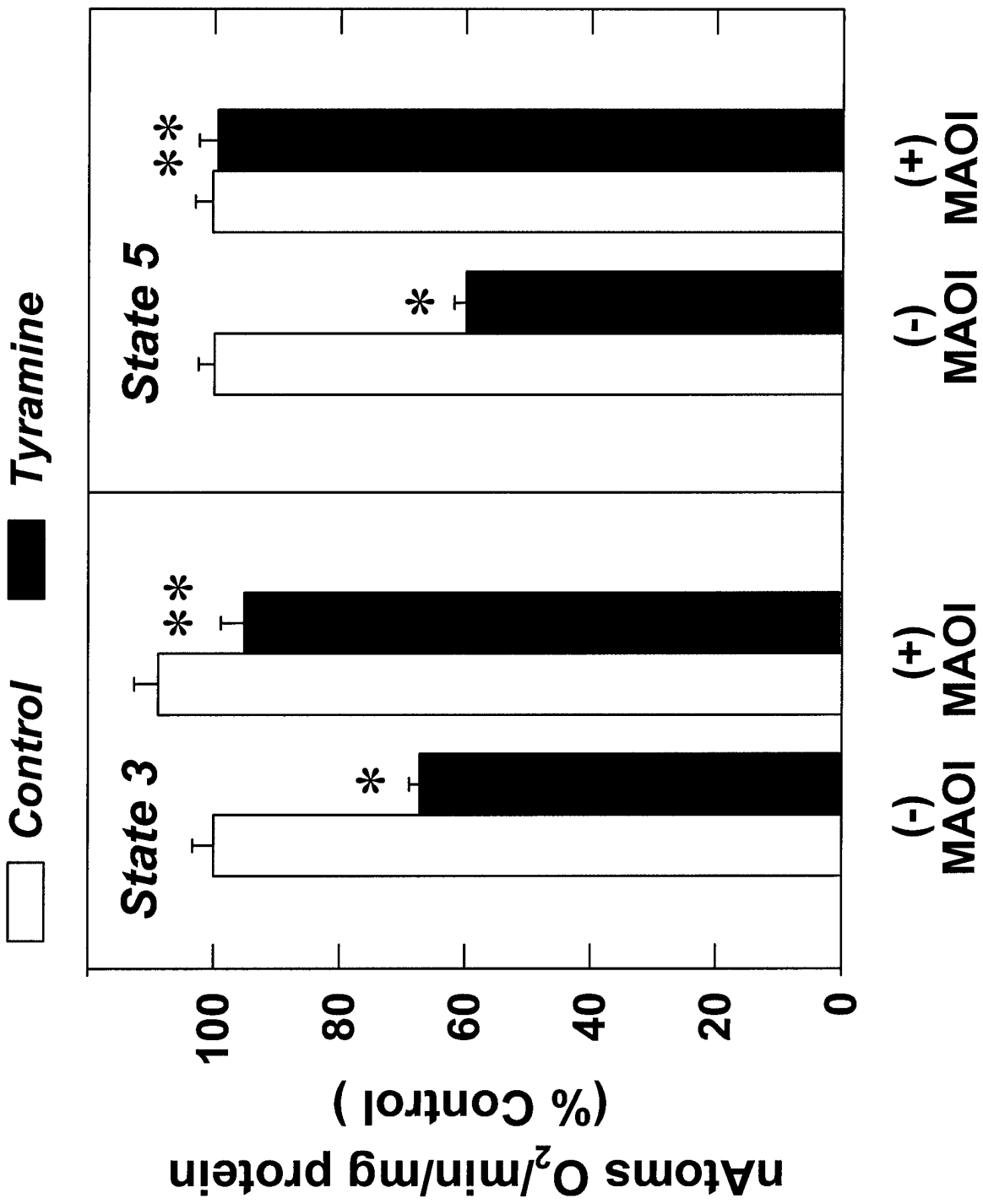

Control
Tyramine
State 3
State 5
nAtoms O2/min/mg protein
(% Control)
100
80
60
40
20
0
*
**
*
**
(-) MAOI
(+) MAOI
(-) MAOI
(+) MAOI

Fig. 2

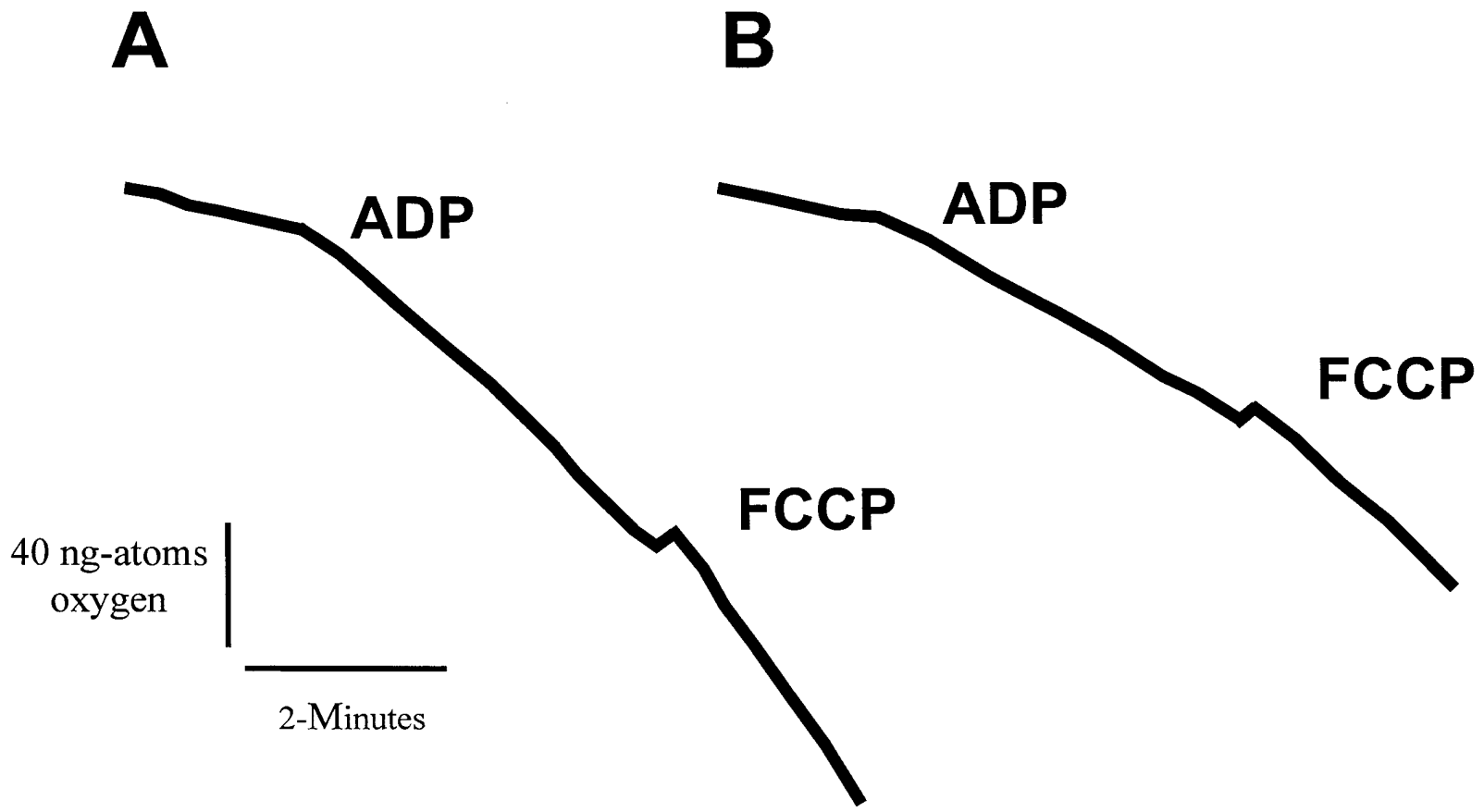


Fig. 3

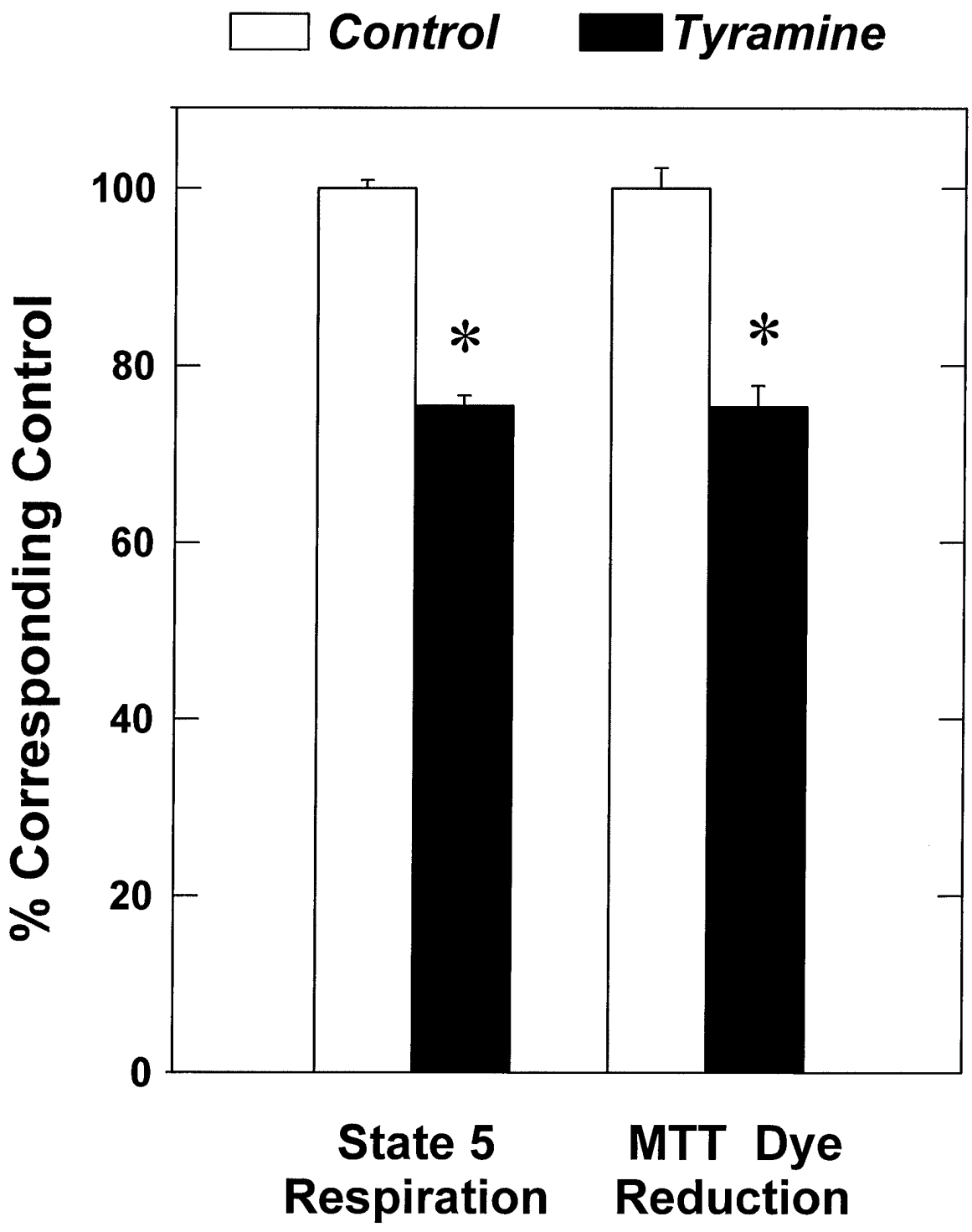

Control
Tyramine
% Corresponding Control
100
80
60
40
20
0
*
*
State 5 Respiration
MTT Dye Reduction

Fig. 4

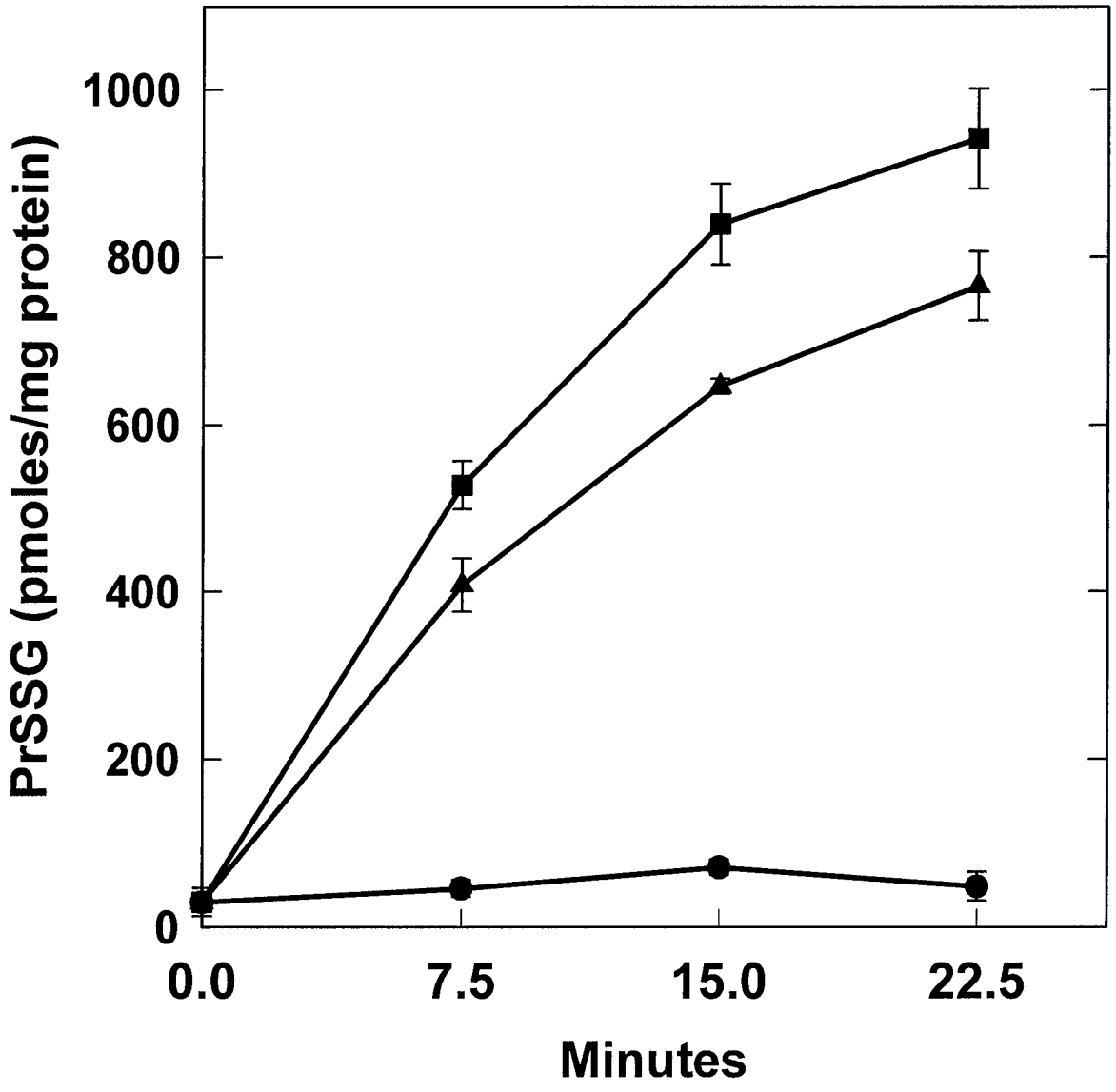
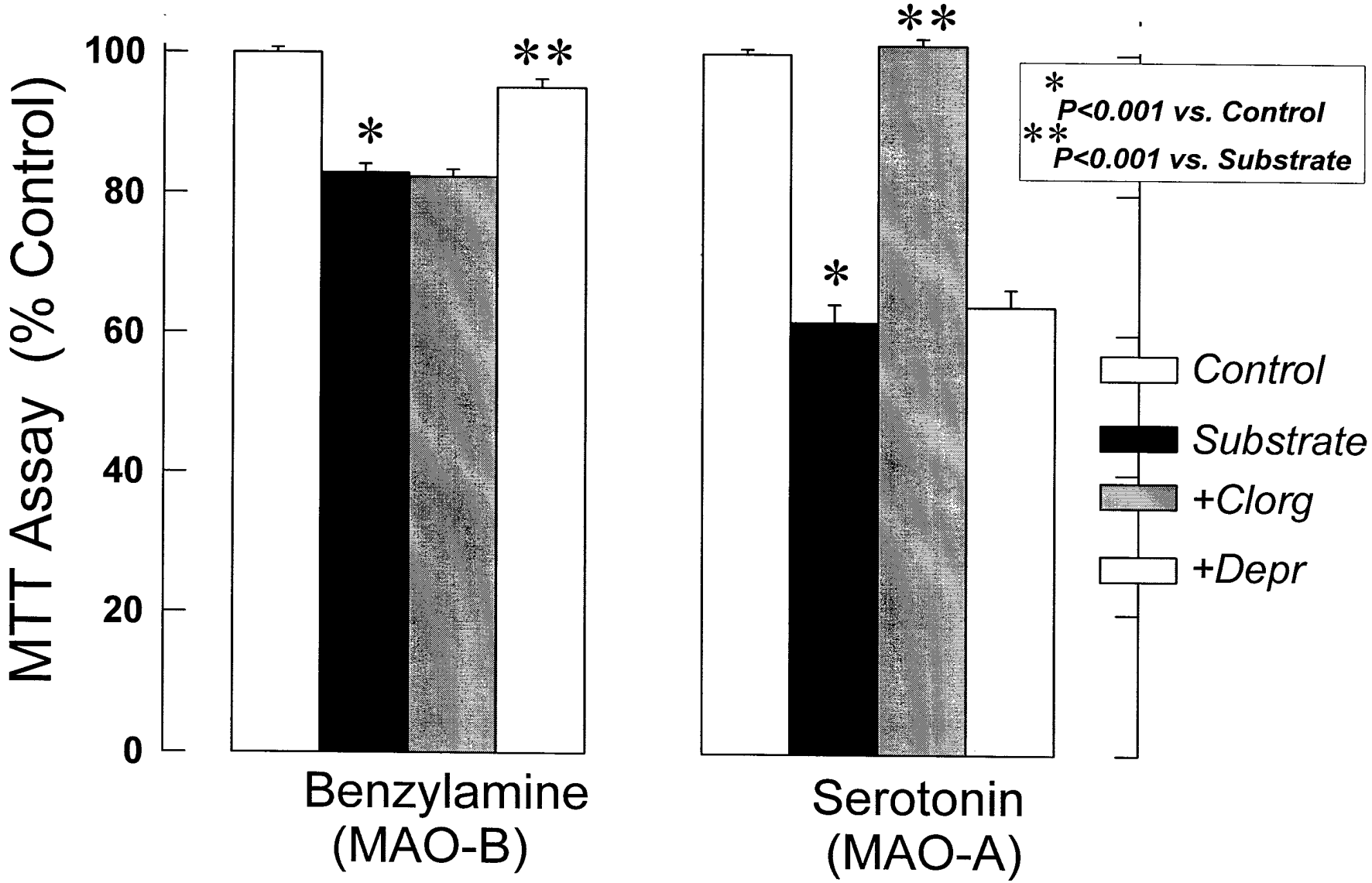

PrSSG (pmoles/mg protein)
1000
800
600
400
200
0
0.0
7.5
15.0
22.5
Minutes

Fig. 5


MTT Assay (% Control)
100
80
60
40
20
0
*
**
*
**
Benzylamine
(MAO-B)
Serotonin
(MAO-A)
* P<0.001 vs. Control
** P<0.001 vs. Substrate
Control
Substrate
+Clorg
+Depr

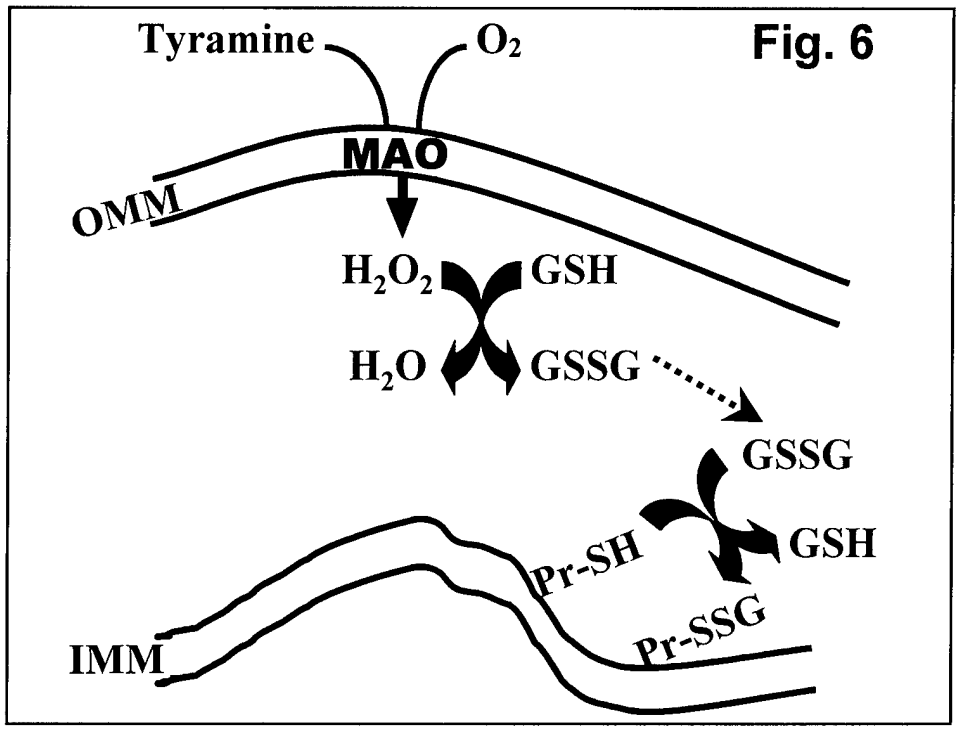


Fig. 6

Fig. 7

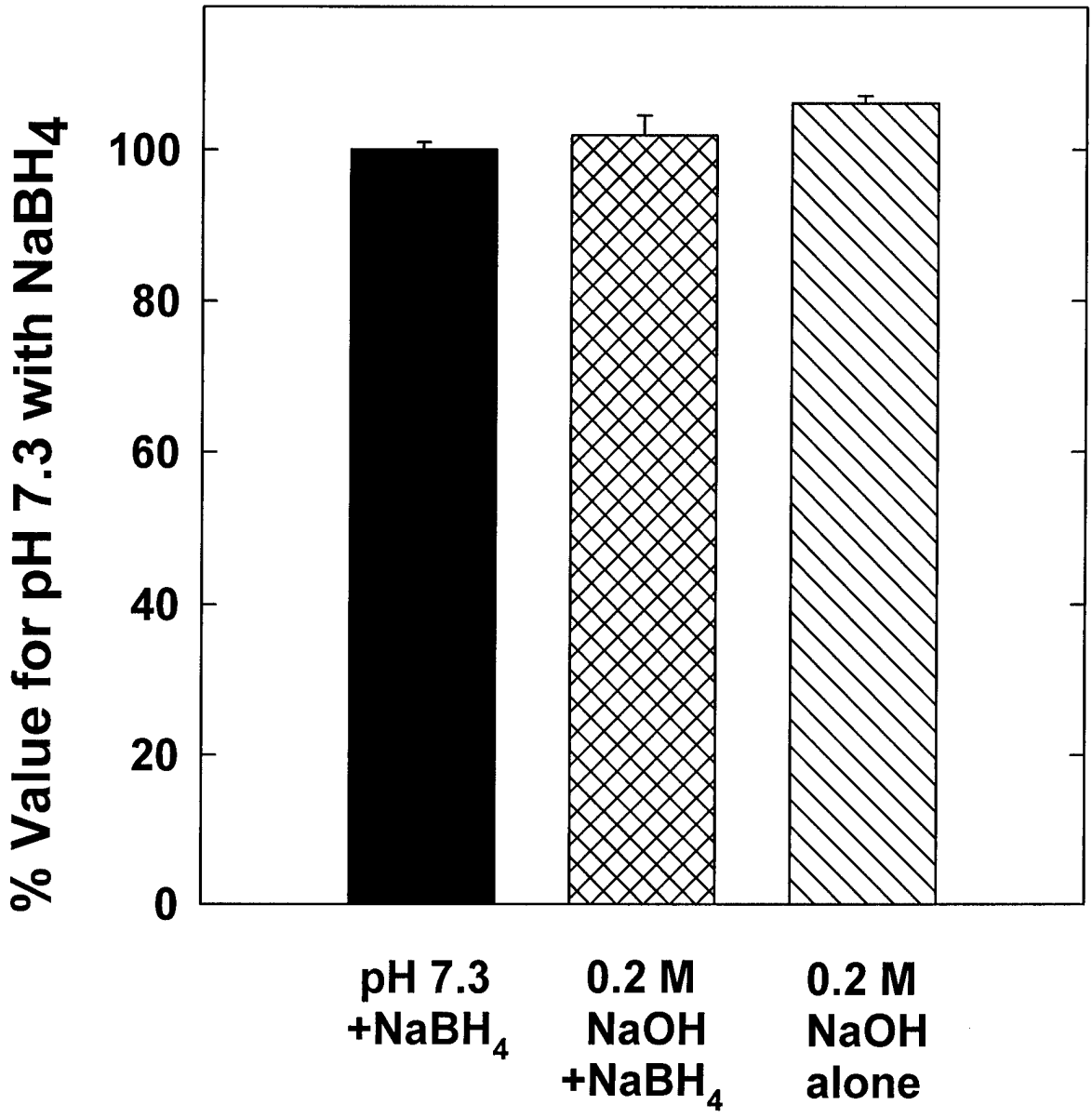
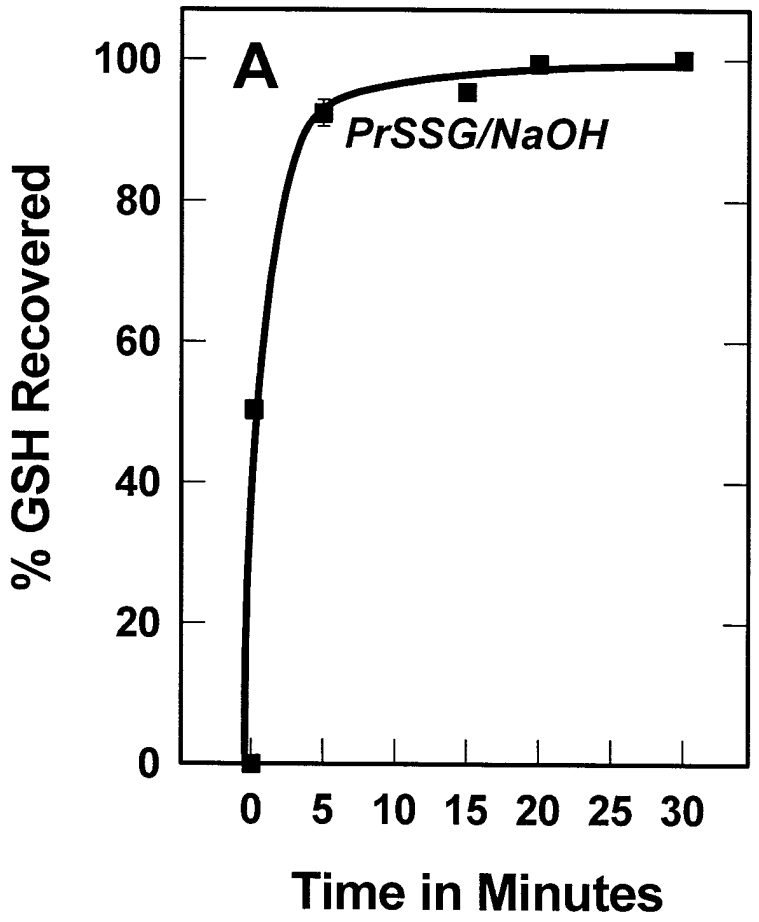

% Value for pH 7.3 with NaBH4
100
80
60
40
20
0
pH 7.3 +NaBH4
0.2 M NaOH +NaBH4
0.2 M NaOH alone

Fig. 8


A
PrSSG/NaOH
% GSH Recovered
100
80
60
40
20
0
0
5
10
15
20
25
30
Time in Minutes

APPENDIX

The appendix, which follows contains 5 Abstracts from meeting presentations, listed by date of presentations:

Cohen G & Kesler N, The link between monoamine oxidase and mitochondrial respiration, 5th Intern. Conf. Parkinson's Disease & Movement Disorders, New York, NY, October 10-14, 1998.

Cohen G & Kesler N., MAO inhibits mitochondrial respiration, 5th Ann. Mtg. Oxygen Society, Washington, DC, Nov. 19-23, 1998.

Cohen G & Yakushin S, Assay of protein-glutathione mixed disulfides, 5th Ann. Mtg. Oxygen Society, Washington, DC, Nov. 19-23, 1998.

Cohen G, Cell signaling and the sulfhydryl redox status, Symposium on Oxidative Stress & Cell Signaling Systems, 30th Ann. Mtg. Amer. Soc. Neurochem., New Orleans, LA, March 13-17, 1999.

Cohen G & Kesler N., Monoamine oxidase and mitochondrial respiration, N.Y. Acad. Sci. Conference on Oxidative/Energy Metabolism in Neurodegenerative Disorders, New York, NY, March 19-22, 1999.

5th INTERNATIONAL CONFERENCE ON PARKINSON'S DISEASE & MOVEMENT DISORDERS, New York, NY. October 10-14, 1998

THE LINK BETWEEN MONOAMINE OXIDASE AND MITOCHONDRIAL RESPIRATION

Gerald Cohen* and Natasa Kesler
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Mount Sinai School of Medicine, New York, NY 10029.

Mitochondria were isolated from rat brain (method of Clark & Nicklas) and incubated at 27°C with substrates for monoamine oxidase (MAO). Subsequently, oxygen consumption was measured during State 3 (ADP-dependent) and State 5 (uncoupled) respiration, supported by pyruvate/malate (5 mM each). MAO substrates, such as dopamine, tyramine, or serotonin, inhibited mitochondrial respiration; however, MAO inhibitors prevented mitochondrial damage. For example, tyramine (500 μM) suppressed State 3 respiration to 67.2 ± 1.7% of control, but inhibition of MAO with clorgyline (2 μM), prior to the addition of tyramine, restored respiration to 95.2 ± 3.7% of control ($p<0.01$). Electron flow, measured by a dye reduction technique with MTT, was similarly suppressed. For example, dopamine (500 μM) suppressed electron flow to 59.2 ± 2.0% of control ($p<0.01$); addition of 2 μM clorgyline restored electron flow to 93.3 ± 1.1% of control ($p<0.01$). When respiration and MTT reduction were directly compared, they gave comparable degrees of inhibition: After exposure to 500 μM tyramine for 15 min., State 5 respiration declined to 75.3 ± 1.2% of control ($p<0.01$) and MTT reduction declined to 75.3 ± 2.4% of control ($p<0.01$). These data show that ADP-stimulated respiration (State 3) and maximally-stimulated respiration (State 5, uncoupled with 10 μM FCCP), as well as the ability to reduce a formazan dye during electron flow (MTT assay), are sensitive to inhibition by MAO-generated H_2O_2. Preliminary evidence indicates that the formation of protein mixed-disulfides (Pr-SSG) may be responsible. In Parkinson's disease, excessive turnover of neurotransmitter by MAO in surviving DA neurons (MAO-A) and associated glia (MAO-B) may contribute to reported deficits in mitochondrial respiration and to the progressive loss of DA neurons.

(Supported by grants from the Parkinson's Disease Foundation & the USAMRMC).

OXYGEN 98: 5TH ANNUAL MEETING OF THE OXYGEN SOCIETY
Washington, DC (Nov. 19-23, 1998)

MAO INHIBITS MITOCHONDRIAL RESPIRATION

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Mount Sinai School of Medicine, New York, NY 10029

Monoamine oxidase (MAO), a mitochondrial H_2O_2-generating enzyme, is responsible for the turnover of neurotransmitter amines, such as dopamine (DA). H_2O_2 is removed by GSH peroxidase, generating glutathione disulfide (GSSG). It is established (Cohen, Farooqui, & Kesler, PNAS, 1997) that MAO activity inhibits mitochondrial electron flow at Complex I, measured by a dye-reduction method (MTT assay). In the current experiments, respiration was directly monitored with an oxygen electrode. Rat brain mitochondria were incubated for 15 min. at 27°C with 500 μM tyramine and, then, State 3 (ADP-coupled) and State 5 (uncoupled, 10 μM FCCP) respiration were measured with pyruvate/malate (5 mM) as substrate. Respiration declined to 60-67% of control ($p<0.01$), but mitochondria were fully protected ($p<0.01$) by 2 μM clorgyline plus pargyline (MAO inhibitors). Additional experiments compared effects on electron flow and respiration: Respiration and the ability to reduce MTT during electron flow were comparably inhibited. Formation of mixed disulfides (PrSSG) with proteins of the respiratory chain may be responsible. Parkinson's disease is associated with a loss of DA neurons in the substantia nigra and diminished Complex I activity. The excessive neurotransmitter turnover, which characterizes the surviving DA neurons, and treatment with L-Dopa, which controls parkinsonian symptoms by elevating brain DA, may be detrimental to mitochondrial respiration and contribute to disease progression.

(Supported by the Parkinson's Disease Fdn.& the USAMRMC).

OXYGEN 98: 5TH ANNUAL MEETING OF THE OXYGEN SOCIETY
Washington, DC (Nov. 19-23, 1998)

ASSAY OF PROTEIN-GLUTATHIONE MIXED DISULFIDES

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Protein-glutathione mixed disulfides (PrSSG) are an indicator of toxic, but reversible, oxidative change to tissue proteins. PrSSG assays are generally based on the release of glutathione (GSH) from protein pellets by reduction with $NaBH_4$ (e.g., Akerboom & Sies, Meth. Enzymol. 77, 1981):

$$PrSSG \rightarrow PrSH + GSH.$$

We have found that simply solubilizing a protein pellet in 0.2 M NaOH gives full release of GSH: 104.3% ± 1.1% (SEM, N=11). Released GSH was measured with a variant of the highly-specific enzymatic recycling assay, with added GSSG reductase, NADPH, & DTNB. Assays were conducted on the supernatant after re-precipitation and re-centrifugation of the protein pellet. Release of GSH by 0.2 M NaOH was remarkably rapid, giving 50% of the maximum yield in 10 seconds at room temperature. Preliminary evidence indicates that the liberation of GSH from PrSSG in alkaline solution can proceed in two ways: (1) rapid reduction of -SS- bonds by protein thiols (PrSH) present in the precipitated protein pellet, and (2) by a slower hydrolysis of disulfide bonds. The spontaneous and rapid release of GSH from isolated protein pellets dissolved in 0.2 M NaOH provides a simple way to assess the accumulation of PrSSG under conditions of oxidative stress.

(Supported by grants from the USPHS & USAMRMC)

AMERICAN SOCIETY FOR NEUROCHEMISTRY, 30TH ANNUAL MEETING
NEW ORLEANS, LA. MARCH 13-17, 1999

SYMPOSIUM ON OXIDATIVE STRESS & CELL SIGNALING SYSTEMS

CELL SIGNALING AND THE SULFHYDRYL REDOX STATUS

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The redox state of thiols can affect cellular regulatory processes. The main tissue thiols are glutathione (GSH, 1-2 mM in brain) and protein sulfhydryls (PrSH, cysteine residues of proteins). Typically, hydrogen peroxide is detoxified by GSH peroxidase (Eqn. 1), resulting in the formation of glutathione disulfide (GSSG). GSSG reacts with proteins to form mixed disulfides (PrSSG, Eqn. 2). This process also leads to the formation of disulfide bridges (PrSSPr, Eqn. 3).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \qquad \text{(Eqn. 1)}$$

$$GSSG + PrSH \longrightarrow PrSSG + GSH \qquad \text{(Eqn. 2)}$$

$$PrSH + PrSSG \longrightarrow PrSSPr + GSH \qquad \text{(Eqn. 3)}$$

The reversible interchange between reduced and oxidized states provides a flexible way to regulate cellular processes. In studies with mesencephalic cell cultures, which contain the dopamine (DA) neurons of the substantia nigra, the addition of DA, apomorphine, or L-Dopa evokes a rise in GSSG, a rise in PrSSG, and up-regulation of cellular GSH. Up-regulation of GSH is accompanied by increased GSH synthesis, which does not require activation of DA receptors. The ability to elevate GSH is shared by compounds that react spontaneously with oxygen to form reactive oxygen species (including catechol and hydroquinone), but not by analogs that do not (e.g., 3-O-methyl-DA, 2,4-dihydroxyphenylalanine). Elevated GSH, in turn, protects cultures against loss of viability in the presence of t-butylhydroperoxide, a powerful oxidant. In an apparent paradox, ascorbate (an antioxidant), blocks the autoxidation of L-Dopa and prevents its antioxidant action. The conclusion is that oxidation is required to evoke the anti-oxidative response. In other experiments, the oxidative deamination of DA by monoamine oxidase, which generates H_2O_2, caused both a rise in mitochondrial PrSSG and suppressed respiration. Thus, oxidant events that alter the sulfhydryl redox status evoke both beneficial and damaging effects.

Supported by grants from USAMRMC and USPHS.

INTERNATIONAL CONFERENCE ON OXIDATIVE/ENERGY METABOLISM IN NEURODGENERATIVE DISORDERS

NEW YORK, NY MARCH 19-22, 1999

Monoamine Oxidase and Mitochondrial Respiration

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Dept. Neurology and Neurobiology Ctr.
Mount Sinai School Medicine, New York, NY 10029

Monoamine oxidase (MAO) is localized to the outer mitochondrial membrane, where it is responsible for the turnover of neurotransmitter amines, such as dopamine (DA), - generating H_2O_2 in the process. H_2O_2, which is potentially toxic, is normally removed by GSH peroxidase. However, a consequence is the formation of glutathione disulfide (GSSG) and protein-glutathione mixed disulfides (PrSSG). We have observed that substrates for MAO possess the ability to inhibit mitochondrial respiration in association with a rise in PrSSG. For example, when rat brain mitochondria are incubated for 15 min. at 27°C with 500 μM tyramine (a mixed MAO-A/MAO-B substrate), State 3 (ADP-coupled) respiration and State 5 (uncoupled, 10 μM FCCP) respiration decline to 60-67% of control ($p<0.01$). However, mitochondria are fully protected ($p<0.01$) by a combination of 2 μM clorgyline plus 2 μM pargyline (selective MAO-A and MAO-B inhibitors, respectively). Similar results were obtained when dopamine was used as substrate and mitochondrial electron flow was measured by a dye reduction assay (MTT assay). Mitochondrial damage by dopamine or tyramine was accompanied by a 6-fold rise in mitochondrial PrSSG. In experiments with serotonin (selective MAO-A substrate), mitochondria were protected by clorgyline (selective MAO-A inhibitor), but not by deprenyl (selective MAO-B inhibitor). On the other hand, mitochondrial damage by benzylamine, a selective MAO-B substrate, was fully prevented by deprenyl, but not by clorgyline. In Parkinson's disease, excessive turnover of neurotransmitter by MAO in surviving DA neurons and associated glia may contribute to deficits in mitochondrial respiration and to the progressive loss of DA neurons.

(Supported by the Parkinson's Disease Fdn. & the USAMRMC).